EMBO reports

The tumor suppressor Brat controls neuronal stem cell lineages by inhibiting Deadpan and Zelda

Ilka Reichardt, François Bonnay, Victoria Steinmann, Inga Loedige, Thomas R. Burkard, Gunter Meister and Juergen A. Knoblich

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 10 April 2017

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge the potential interest of the findings. However, all referees also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed. None of the referees is fully convinced that the current set of data is sufficient to support the proposed model of tumor formation and cell specification. Moreover, referee 1 points out that the conclusions rely exclusively on RNAi knockdown and also referee 3 agreed upon further discussion that the findings should be strengthened using mutant alleles.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section

called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

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REFEREE REPORTS

Referee #1:

Brain tumor (Brat) plays a key role in regulating neural stem cell (neuroblast) differentiation in fly brains, but the mechanisms by which this tumor suppressor carries out its function remain unclear. Two recent studies by Laver et al. and Loedige et al. strongly suggest that Brat can regulates gene expression by binding to a specific motif in the 3'UTR of its target mRNAs in both fly embryos and in fly brain neuroblasts. The study by Reichardt et al. expanded this line of evidence by showing that Brat likely promotes the differentiation of the neuroblast progeny, imINP, by down-regulating the expression of Deadpan and Zelda through translational repression. By analyzing a novel Brat allele, which results in mis-segregation of Brat and differential regulation of Deadpan and Zelda expression in imINPs, the authors propose a model where repression of Dpn expression in imINPs requires a higher level of Brat activities than the repression of Zld expression. The authors speculate that a lower level of Brat activities prevent re-expression of Zelda in mINPs.

Data showing the requirement of Zelda for the brain tumor phenotype in the Brat mutant genetic background and analyses of a novel Brat mutant allele are interesting and support the model that a lower level of Brat is sufficient for the initial down-regulation of Zelda expression in imINP. However, the other half of their model where a lower level of Brat activities prevents the re-expression of Zeld in mINPs is not well supported by their data. In addition, I am concerned about the strategy employed in obtaining the function data solely relied on RNAi knock down, and the of Zelda in promoting the re-entry into cell cycle in imINPs. Therefore, I can fully support publication of this manuscript in its current state.

Major concerns:
1. The authors provide strong correlative data supporting their hypothesis that Brat represses the expression of Zelda in imINPs through binding to its 3'UTR. However, direct evidence demonstrating this mechanistic link in a physiological context is missing from this study. Please demonstrate that Brat-binding to the 3'UTR of Zelda transcripts is indeed required for down-regulation of Zelda expression in imINPs.

2. The authors should verify the Zelda and Deadpan knock-down phenotypes that they reported in this study using available genetic loss-of-function alleles. In addition, the authors should carry rescue experiments to confirm that the knock-down phenotypes can be suppressed by re-storing Zelda and Deadpan expression.

3. I am still uncertain about the exact function of Zelda in the type II neuroblast lineage. The authors conclude that Zelda expression is mostly detected in neuroblasts but is not required for maintaining neuroblast self-renewal. In addition, the authors also conclude that Zelda is not expressed in many imINPs and mINPs, but is required for re-expression of Deadpan in mINPs and for re-initiation of the cell cycle of imINPs and mINPs. To complicate the interpretation further, it is clear that two distinct isoforms of Zelda is transcribed in cells of the type II neuroblast lineage. Please reconcile all of the seemingly contradictory observations, and explain how the authors rule out the possibility that some of these phenotypes are associated to the loss of the PD isoform, which appears to be transcribed in imINPs and mINPs.

4. I remain skeptical regarding the explanation provided by the authors regarding decreased mINPs in Zelda knock-down brains. How can the authors rule out the possibility that a decreased number of mINPs is not due to slow-down of maturation and a decreased number of Ph3+ mINPs is not due to cell cycle slow-down? Perhaps more importantly, how can this defect contribute to brain tumor formation in Brat mutant brains.

5. The authors should direct compare the level of Brat expression in neuroblasts and in mINPs to first convince the readers that there is indeed a lower level of Brat in mINPs. Does knocking down Brat function in imINPs and mINPs lead to ectopic Zelda expression in mINPs? If not, the authors should tone down their conclusion and propose alternative model.

Minor concerns:
1. The description of for bratRNAi and dpnRNAi shown in Figure S2 is not present in the text.

2. Data shown in Figure 3g-h are overlapping to with those in Figure S4b-c. In addition, the description for Figure 3g-h in unavailable in the text.

3. The data showing repression of Deadpan expression in bratG860D brains are absent in Figure S7b.

Referee #2:

Stem cells balance self-renewal with differentiation and misregulation thereof can cause stem cell overgrowth and tumorigenesis. Drosophila neuroblasts, the neural stem cells of the fly are a well-suited system to investigate the regulation of stem cell self-renewal and differentiation. Neuroblasts can be subdivided into type I and type II, the later forming a transit amplifying pool of intermediate neural precurors (INPs). All neuroblasts express the transcription factor Deadpan, but newly born, immature INPs first switch of Dpn expression before turning on the expression of the transcription factor Asense (Ase). Mature INPs (mINPs) reinitiate the expression of Dpn.
Previously it was shown that in brat mutants, immature INPs fail to initiate Ase and Dpn expression and revert back to type II Nbs, creating excessive neuroblasts and tumor formation. The molecular basis of how Brat acts in neuronal lineages is not entirely clear. Here, the Knoblich lab identified Zelda (Zld), a zinc-finger protein as a translational target of Brat, binding directly to zelda's 3' UTR with high affinity. Reichardt et al. further show that Brat also affects the stability of dpn transcripts by binding to dpn's 3'UTR with lower affinity. The finding that Brat binds zld and dpn with different affinity let Reichardt et al. to propose that different Brat concentrations regulate the repression of zld and dpn, thereby regulating the progression of type II lineage.

Overall, this is a solid manuscript, providing novel insight into fate generation in the type II neural stem cell lineage. Thus, it is well suited for EMBO reports.
However, the authors should address the following concerns before the paper can be considered fit for publication.

General comments:

1. The authors identify mRNA changes after knocking-down Brat in type II neuroblast lineages and analyzed the transcriptome 24h after brat RNAi induction. At this point, cell numbers are still the same as in wild type. The provided images in Figure 1b are inadequate to demonstrate the phenotype

of brat knock-down at the different time points; highlights of individual neuroblasts and their progeny would improve the manuscript.

2. It is not quite clear why the authors decided to follow up on the characterization of Zelda; according to the data provided in Figure 1c, the changes in zld mRNA are at the lower end compared to other genes. Also, it would be beneficial to highlight zld in the graph in Figure 1c; it is currently still labelled with vfl.

3. p.7: the authors state: "we quantified brat tumor formation by Western Blot analysis of Miranda and type II Nb specific GFP expression and observed a significant tumor reduction in brat zld double RNAi compared to brat RNAi (Fig.2a). This sentence does not quite make sense. Although the western blot does show a reduction in both Mira and GFP signal in the double RNAi compared to brat, it does not show a reduction in tumor formation.

4. p.7: The authors transplant brain tissue of brat and brat zld double RNAi into the thorax of wild type host flies to characterize whether Zld is a tumor promoting factor. To my knowledge, in the classical tumor transplantation assay, tissue is grafted into the abdomen of wild type hosts and not the thorax. Why did the authors transplant tissue into the thorax and not the abdomen? Do abdominal transplantations not result in tumor formation?

5. p. 7, Fig.2c: the authors state that upon brat zld double RNAi, ectopic NBs expressed both Dpn and Ase or even Ase alone. The provided overview panels in Fig.2c make it difficult to see follow this phenotype in individual neuroblast lineages. Also, whether the brat zld knock-down specifically affects type II neuroblast lineages is unclear and should be clarified.

6. Along the same lines, the manuscript would greatly improve if it would contain a panel allowing to compare a representative wild type with a brat mutant, zld single mutant and brat zld double mutant type II neuroblast lineage in respect of Dpn and Ase expression. For instance, the provided panel in Suppl. Fig. 3a could be expanded with all the different mutant and double mutant conditions.

7. p.8 and Figure 3. The authors use FISH and Zld::V5 to determine zelda's expression and localization, resulting in the conclusion that it is exclusively present in Nb and quickly degraded in imINPs. How can the authors exclude that induction/maintenance of zld expression is lower in imINPs, and that the lack of zld transcript and Zld protein is independent of its stability?

8. What is the phenotype of brat dpn double mutants?

9. Ultimately, it is unclear to me how Zld expression correlates with tumor formation. bratG774D mutants apparently don't form brain tumors but can repress zld-PB but not Dpn. Does that mean that failure to repress Dpn expression is not sufficient for tumor formation? I thought that increase of Dpn reverts INPs to type II Nbs?

Minor:
p. 9: Third line: The sentence should read "Second, in order to...".
Figure 3h: This panel seems to be duplicated and is also shown in Supplemental Figure S4.

Referee #3:

This study addresses the role of the TRIM-NHL protein Brat in controlling Type II neuroblast development in the Drosophila larvae brain. Brat mutants show extensive brain overgrowth, and the authors aimed to understand the molecular function of Brat. They find that a small group of genes are upregulated in brat RNAi brains, 5 of which encode transcription factors. Suppressor tests for lethality point to two of the 5 TFs as particularly important: Zelda and Dpn. They find that RNAi of zelda and dpn in part phenocopy brat, and go on to decode how brat controls these genes. They find that Brat binds to specific sequences in the 3´UTR of both the zelda and dpn RNAs. Presumably due to ten Brat-target motifs in zelda and four in dpn, the negative regulation of zelda is more sensitive to Brat levels. This notion is partly verified by analysis of point-mutants of brat, and mis/overexpression of wt brat and mutant brat constructs. The negative regulation of translation by

Brat onto the 3´UTR sequences can be re-capitulated also on a heterologous GFP construct in S2 cells.
I think this study provides an impressive push towards decoding the molecular underpinning of the brat brain overgrowth phenotype, and given the interest in TRIM proteins in many systems, including mammals, I think the story should be of broad interest. I have a few issues that may help strengthen the story:

Major issues:
1) Their transcriptome data, showing up-regulation of zelda and dpn mRNAs, point to a model where Brat binding results in degradation of zelda and dpn mRNAs. Is this the only way Brat acts? Or does Brat binding somehow inhibit mRNA nuclear export, ribosome docking and/or elongation?

2) What are the downregulated genes identified in the transcriptome analysis? They do not comment on this. They should show table of these genes.

3) In the same vein: What is the basis for increased proliferation in brat, Zelda and dpn RNAi or mutants? Increased CycE, Stg or E2f1, decreased Dap? Amongst the top upregulated genes, I could not recognize any clear cell cycle gene. Are they showing up in the downregulated genes?

4) What is the phenotype of the brat[G774D] mutant? There is still repression of zld but dpn is ectopically expressed. But what is the consequence of this with regards to the Type II lineage development? PH3-labeling? Clone size?

Minor issues:
5) Abstract: Drosophile should be Drosophila.
6) Introduction, 2nd paragraph: Drosophila NBs also come in the Type 0 flavor (PMIDs 15593370, 19945380, 25073156, 25171415).
7) I think the model in the supplement (S8) could be moved into the final main figures. I think their data presented here, combined with previous results, justify highlighting this model inside the main paper.
8) In several figure legends (main text and suppl) Granyhead should be Grainy head or Grh. Also in the main text: Grainyhead should be Grainy head or Grh.
9) Supplemental Figure S4e: Maybe help the reader along by outlining the green lineages in the panels to help show that V5 staining is lost in the zld-RNAi expressing lineages.
10) Pages 8-9: I do not agree with the notion of "active" and "inactive" zelda transcripts. What they have are two different splice variants. If they want to push the notion of active-inactive, they should use RNAi directed against each isoform separately, and/or use Crispr/Cas9 to mutate each isoform separately. I understand that previous studies have pointed to these roles of zelda isoforms, but this was in a different system.
11) Figure 4c-d: In the EMSA with Brat and Zelda RNA, why do the bound complexes migrate at different positions depending on the amount of Brat protein?
12) There are a bunch of typos throughout, and the manuscript would benefit from some closer scrutiny.

1st Revision - authors' response 30 August 2017

Referee #1:

Brain tumor (Brat) plays a key role in regulating neural stem cell (neuroblast) differentiation in fly brains, but the mechanisms by which this tumor suppressor carries out its function remain unclear. Two recent studies by Laver et al. and Loedige et al. strongly suggest that Brat can regulates gene expression by binding to a specific motif in the 3'UTR of its target mRNAs in both fly embryos and in fly brain neuroblasts. The study by Reichardt et al. expanded this line of evidence by showing that Brat likely promotes the differentiation of the neuroblast progeny, imINP, by down-regulating the expression of Deadpan and Zelda through translational repression. By analyzing a novel Brat allele, which results in mis-segregation of Brat and differential regulation of Deadpan and Zelda expression in imINPs, the authors propose a model where repression of Dpn expression in imINPs requires a higher level of Brat activities than the repression of Zld expression. The

authors speculate that a lower level of Brat activities prevent re-expression of Zelda in mINPs.

Data showing the requirement of Zelda for the brain tumor phenotype in the Brat mutant genetic background and analyses of a novel Brat mutant allele are interesting and support the model that a lower level of Brat is sufficient for the initial down-regulation of Zelda expression in imINP. However, the other half of their model where a lower level of Brat activities prevents the re-expression of Zeld in mINPs is not well supported by their data. In addition, I am concerned about the strategy employed in obtaining the function data solely relied on RNAi knock down, and the of Zelda in promoting the re-entry into cell cycle in imINPs. Therefore, I can fully support publication of this manuscript in its current state.

Major concerns:
1. The authors provide strong correlative data supporting their hypothesis that Brat represses the expression of Zelda in imINPs through binding to its 3'UTR. However, direct evidence demonstrating this mechanistic link in a physiological context is missing from this study. Please demonstrate that Brat-binding to the 3'UTR of Zelda transcripts is indeed required for down-regulation of Zelda expression in imINPs.

> We agree that this experiment would have been a great addition to our current S2 cells results. The most straightforward way to demonstrate this point would have been to mutate the various Brat binding sites from the 3'UTR of Zelda with CRISPR/Cas9. This would have required a long genome engineering process with very uncertain outcome: we do not know whether such flies would survive, given the fundamental role of zld in the embryo. The alternative of generating a GFP reporter line carrying *zld* 3'UTR would be hard to analyze in vivo, since the GFP expressed from the NB/imINP would remain present in the INPs with a different turnover than Zld itself. For all these reasons, we did not perform the requested experiment.

2. The authors should verify the Zelda and Deadpan knock-down phenotypes that they reported in this study using available genetic loss-of-function alleles. In addition, the authors should carry rescue experiments to confirm that the knock-down phenotypes can be suppressed by re-storing Zelda and Deadpan expression.

> We followed reviewer's suggestions and performed two rescue experiments: (1) we re-introduced *Zld-RB* into zld^{shmiR} (targeting 3'UTR of *Zld-RB*) type II NB lineages and could restore the appearance of Dpn+ INPs (new suppl. Fig. S4a); (2) we over-expressed Dpn in a zld^{shmiR}, $brat^{RNAi}$ tumor context and could restore the full growth of the tumor (new suppl. Fig S4b-d).
Concerning reviewer's first point, we obtained commercially available Kyoto DGRC zelda loss-of-function lines 111874 (FRT19A, zld^{G0353}) and 111837 (FRT19A, zld^{G0427}) but could not obtain consistent and interpretable loss of function data. Instead, our results using those alleles (complete loss of type II NB lineages) suggested that there are second-site mutations on those chromosomes that make the interpretation of these experiments impossible in our system. We also made numerous attempts to generate new Zelda alleles using CRISPR/Cas9 but were not successful. As such alleles are routinely generated in our laboratory, we conclude that some specific features of the Zelda locus make it inaccessible for the Cas9 nuclease. However, we want to highlight that the *zld* phenotype is now depicted by a total of four independent RNAi constructs: one shmiR and one inverted repeat construct directed against all isoforms, two shmiR directed specifically against *zld–RB*.

3. I am still uncertain about the exact function of Zelda in the type II neuroblast lineage. The authors conclude that Zelda expression is mostly detected in neuroblasts but is not required for maintaining neuroblast self-renewal. In addition, the authors also conclude that Zelda is not expressed in many imINPs and mINPs, but is required for re-expression of Deadpan in mINPs and for re-initiation of the cell cycle of imINPs and mINPs. To complicate the interpretation further, it is clear that two distinct isoforms of Zelda is transcribed in cells of the type II neuroblast lineage. Please reconcile all of the seemingly contradictory observations, and explain how the authors rule out the possibility that some of these phenotypes are associated to the loss of the PD isoform, which appears to be transcribed in imINPs and mINPs.

> We followed reviewer's suggestions and performed isoform-specific knock-down of the *–RB* and *–RD* isoform with four new constructs in *brat* tumor settings. While *zld-RB* specific shmiR led to rescued *brat* tumor, none of the three *zld-RD* shmiRs did. Interestingly, *zld-RD* specific shmiRs

instead showed a mild positive effect on tumor growth (new suppl. SFig 5a-c). Together with the rescue experiment performed with *zld-RB* (new suppl. SFig 4a), these results indicate that the *zld-RB* isoform is key for proliferative ability of type II NB lineages, while *zld-RD* is dispensable.

4. I remain skeptical regarding the explanation provided by the authors regarding decreased mINPs in Zelda knock-down brains. How can the authors rule out the possibility that a decreased number of mINPs is not due to slow-down of maturation and a decreased number of Ph3+ mINPs is not due to cell cycle slow-down? Perhaps more importantly, how can this defect contribute to brain tumor formation in Brat mutant brains.

> Importantly, while PH3+ mINPs number was lower in *zld* knock-down conditions, the total number of INPs was similarly lower (Fig 3c, suppl. Fig S6d-f), therefore indicating that a smaller number of INPs divide with similar kinetics between WT and *zld* deficient type II NB lineages. A slow-down of INPs maturation would, in principle, lead to an increased representation of early INP temporal patterning such as Dichaete+ or Grh+, which we didn't observe (Fig 3c-d, suppl. Fig S6d-f). Our hypothesis is that the progeny of *zld*-deficient type II NB undergoes lower number of cell cycle before differentiation, due its failure to re-express *dpn*. Repressing *dpn* specifically in INPs led to the same phenotype (Fig 3a-b), which further supports this hypothesis. In a *brat* context, *zld* deficient type II NB progeny would encounter a similar defect in expressing dpn, even in the absence of *brat*–mediated repression, therefore limiting its tumor growth potential.

5. The authors should direct compare the level of Brat expression in neuroblasts and in mINPs to first convince the readers that there is indeed a lower level of Brat in mINPs. Does knocking down Brat function in imINPs and mINPs lead to ectopic Zelda expression in mINPs? If not, the authors should tone down their conclusion and propose alternative model.

> We followed reviewer's suggestion to follow Zelda expression levels in mINPs. Since knocking down of *brat* in imINPs lead to tumor formation and therefore no-longer mINPs maturation, we knocked-down *brat* in mINPs that we isolated by FACS with GFP under the control of erm>Gal4 (3^{rd} chromosome). Both qPCR on this mINPs population and immunostainings (already present in Figure 6c-d in the former version of the manuscript) showed an increase in Zelda RNA and protein levels upon *brat* RNAi in mINPs (Figure 6c-e). Interestingly, we could also observe a significant increase of Dpn RNA levels upon *brat* RNAi in mINPs (Figure 6e).
Concerning reviewer's first point, the most significant difference in Brat levels we could observe occurred between imINPs and mINPs which we quantify in suppl. Figure S9b-c. NB-Brat is cortical and systematically segregated out of the NB after each cell division which makes it difficult to visualize and to quantify (suppl. Fig S9b).

Minor concerns:

1. The description of for bratRNAi and dpnRNAi shown in Figure S2 is not present in the text.
> We apologize for this mistake. We have now added a description of these panels in the main text as followed: "As a positive control, *brat dpn* double RNAi adults developed reduced tumors to an even higher extent, further confirming the importance of Dpn expression in *brat* tumor growth (suppl. Fig. S3)."

2. Data shown in Figure 3g-h are overlapping to with those in Figure S4b-c. In addition, the description for Figure 3g-h in unavailable in the text.
> We apologize for this mistake. We have now removed Figure 3g-h panels and have added the localization of the FISH probes originally present in Figure 3g to the Supplement Figure S7b (formerly S4b).

3. The data showing repression of Deadpan expression in bratG860D brains are absent in Figure S7b.
> We apologize for this mistake and have added $Brat^{G860D}$ (showing no Dpn repression) type II NB lineages to the panel (now suppl. Fig S10b).

Referee #2:

Stem cells balance self-renewal with differentiation and misregulation thereof can cause stem cell

overgrowth and tumorigenesis. Drosophila neuroblasts, the neural stem cells of the fly are a well-suited system to investigate the regulation of stem cell self-renewal and differentiation. Neuroblasts can be subdivided into type I and type II, the later forming a transit amplifying pool of intermediate neural precurors (INPs). All neuroblasts express the transcription factor Deadpan, but newly born, immature INPs first switch of Dpn expression before turning on the expression of the transcription factor Asense (Ase). Mature INPs (mINPs) reinitiate the expression of Dpn.

Previously it was shown that in brat mutants, immature INPs fail to initiate Ase and Dpn expression and revert back to type II Nbs, creating excessive neuroblasts and tumor formation. The molecular basis of how Brat acts in neuronal lineages is not entirely clear. Here, the Knoblich lab identified Zelda (Zld), a zinc-finger protein as a translational target of Brat, binding directly to zelda's 3' UTR with high affinity. Reichardt et al. further show that Brat also affects the stability of dpn transcripts by binding to dpn's 3'UTR with lower affinity. The finding that Brat binds zld and dpn with different affinity let Reichardt et al. to propose that different Brat concentrations regulate the repression of zld and dpn, thereby regulating the progression of type II lineage.

Overall, this is a solid manuscript, providing novel insight into fate generation in the type II neural stem cell lineage. Thus, it is well suited for EMBO reports.

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General comments:

1. The authors identify mRNA changes after knocking-down Brat in type II neuroblast lineages and analyzed the transcriptome 24h after brat RNAi induction. At this point, cell numbers are still the same as in wild type. The provided images in Figure 1b are inadequate to demonstrate the phenotype of brat knock-down at the different time points; highlights of individual neuroblasts and their progeny would improve the manuscript.

> We have followed this important suggestion and added zoomed pictures of Figure 1b in new suppl. Fig. S2. Type II NBs and the different fate of their progeny have been labelled directly on the picture: imINP and mINP in control lineages; Dpn+ imINP and ectopic neuroblast in a $brat^{RNAi}$ lineages.

2. It is not quite clear why the authors decided to follow up on the characterization of Zelda; according to the data provided in Figure 1c, the changes in zld mRNA are at the lower end compared to other genes. Also, it would be beneficial to highlight zld in the graph in Figure 1c; it is currently still labelled with vfl.

> Zelda was indeed not one of the most upregulated gene in $brat^{RNAi}$ tumor initiating cells. However, our interest in studying Zelda was further motivated by the study of Laver et al., 2015 describing *zld* as one of the key target of Brat during maternal-to-zygotic transition. Even more importantly, *zelda* knock-down gave, after *deadpan*, the most potent rescue of $brat^{RNAi}$-induced lethality assay (Figure 1e).
vfl was accordingly corrected into zld and highlighted in bold blue in Figure 1c.

3. p.7: the authors state: "we quantified brat tumor formation by Western Blot analysis of Miranda and type II Nb specific GFP expression and observed a significant tumor reduction in brat zld double RNAi compared to brat RNAi (Fig.2a). This sentence does not quite make sense. Although the western blot does show a reduction in both Mira and GFP signal in the double RNAi compared to brat, it does not show a reduction in tumor formation.

> We agree with the reviewer. Our results indicate that zelda is required for the growth capacity rather than the *formation* of *brat* originated tumors. We reformulated our statement accordingly: “we quantified brat tumor growth […]”.

4. p.7: The authors transplant brain tissue of brat and brat zld double RNAi into the thorax of wild type host flies to characterize whether Zld is a tumor promoting factor. To my knowledge, in the classical tumor transplantation assay, tissue is grafted into the abdomen of wild type hosts and not

the thorax. Why did the authors transplant tissue into the thorax and not the abdomen? Do abdominal transplantations not result in tumor formation?

> Abdominal transplantations of brain tumor pieces indeed result in the formation of metastasis but are harmful and suffer from a significant level of variability. These assays can provide a qualitative answer of tumor formation ability but do not perform well in quantifying the efficiency of metastasis formation comparing differently growing tumors, since the amount of injected tumor material cannot be properly controlled. To test more precisely the role of Zelda as a tumor-promoting factor, we developed a transplantation assay allowing the injection of a precise amount of tumor cells (in this study, 500 tumor cells per fly were injected) and therefore a more controlled metastasis assay. This technique allowed us to inject a precise volume of concentration-defined tumor cell suspension that led to 100% efficiency of metastasis in control tumors. The injection was performed with a Nanoject II device functioning with capillaries that cannot penetrate easily the abdomen due to their thinner diameter. The transplantation was instead performed in the thorax in an easily-penetrable "weak spot" between the wing and the "shoulders" to avoid significant injury. We are currently preparing a technical manuscript describing this new transplantation method.

5. p. 7, Fig.2c: the authors state that upon brat zld double RNAi, ectopic NBs expressed both Dpn and Ase or even Ase alone. The provided overview panels in Fig.2c make it difficult to see follow this phenotype in individual neuroblast lineages. Also, whether the brat zld knock-down specifically affects type II neuroblast lineages is unclear and should be clarified.

> We've followed reviewer's suggestion and are now providing zoomed images depicting *brat zld* double RNAi tumor cells compared to single mutants with Dpn and Ase stainings (suppl. Fig. S6a). The knock-down of *brat* and *zld* shown in Fig.2C was performed with the type II NB-specific driver Wor-Gal4, Ase-Gal80, as stated in the Figure legends. We've now added this information directly on the panel.

6. Along the same lines, the manuscript would greatly improve if it would contain a panel allowing to compare a representative wild type with a brat mutant, zld single mutant and brat zld double mutant type II neuroblast lineage in respect of Dpn and Ase expression. For instance, the provided panel in Suppl. Fig. 3a could be expanded with all the different mutant and double mutant conditions.

> We've followed reviewer's suggestion and have added to Suppl. Fig. S6a (former Suppl. Fig. S3a) two sets of panels zooming on individual type II NB "lineages" (it is hard to delimitate an individual lineage in a *brat* tumor context, we rather delimitated clusters of neighboring cells) from *brat* RNAi single and *brat* RNAi, *zld* IR double RNAi conditions. These new panels now better illustrate the appearance of Ase+ cells in *brat* IR, *zld* IR rescued tumors.

7. p.8 and Figure 3. The authors use FISH and Zld::V5 to determine zelda's expression and localization, resulting in the conclusion that it is exclusively present in Nb and quickly degraded in imINPs. How can the authors exclude that induction/maintenance of zld expression is lower in imINPs, and that the lack of zld transcript and Zld protein is independent of its stability?

> We thank the reviewer for this suggestion. We'd like to point out that imINPs are a very rare and hard-to-isolate population of cells, that, to our knowledge, was so far never studied differently than with immuno-staining. We tried to isolate these cells with a combination of Type-II NB specific Gal4 and INP-specific LexA-VP16 drivers (sorting Gal4-RFP positive, LexA-VP16-GFP negative cells). We were unfortunately unable to FACS-sort a pure population of imINPs and repeatedly experienced contamination with other NB subtypes, likely optic-lobe NBs which have a similar size, expressing high amounts of both Dpn and Zld. We therefore cannot exclude the possibility that Zld is transcriptionally regulated differently between NB and imINPs in addition to brat-mediated repression. This has been now highlighted in the discussion: "Importantly, our data do not exclude that pre- or co-transcriptional regulation of these factors would occur in parallel of Brat-mediated post-transcriptional control".

8. What is the phenotype of brat dpn double mutants?

> We depict *brat dpn* double RNAi type II NB phenotype in suppl. Fig S3 and in new suppl. Fig S5a-c. Depleting *dpn* in type II NB strongly affected their proliferation capacity and therefore largely prevented tumor formation.

9. Ultimately, it is unclear to me how Zld expression correlates with tumor formation. bratG774D mutants apparently don't form brain tumors but can repress zld-PB but not Dpn. Does that mean that failure to repress Dpn expression is not sufficient for tumor formation? I thought that increase of Dpn reverts INPs to type II Nbs?

> We further followed *brat*^{G774D} mutant clones after 72h and still could not observe reversion into ectopic or general over-proliferation, despite the lack of Dpn repression in the first imINPs (Suppl. Fig. S8f-g). Instead, these INPs were able to give rise to Dpn- differentiated progenies similarly to WT. We believe that other factors than Dpn with high affinity to Brat-mediated repression are required for tumor formation. In addition to Zld, our brat tumor survival rescue analysis points to Grh, Myc and Pnt as potential candidates (Fig. 1e).

Minor:
p. 9: Third line: The sentence should read "Second, in order to...".
> This has been corrected.

Figure 3h: This panel seems to be duplicated and is also shown in Supplemental Figure S4.
> We apologize for this mistake. The reviewer is right, there were two panels' ensembles (Fig3h and suppl. Fig. S4c) that were exactly similar (all 5 panels have the exact same labeling in the two contexts), but importantly, they did not deliver separate messages and were not used separately in the text. It happened when reordering the figures/supplementaries during the first submission process.
We have corrected this by removing Figure 3h panel.

Referee #3:

This study addresses the role of the TRIM-NHL protein Brat in controlling Type II neuroblast development in the Drosophila larvae brain. Brat mutants show extensive brain overgrowth, and the authors aimed to understand the molecular function of Brat. They find that a small group of genes are upregulated in brat RNAi brains, 5 of which encode transcription factors. Suppressor tests for lethality point to two of the 5 TFs as particularly important: Zelda and Dpn. They find that RNAi of zelda and dpn in part phenocopy brat, and go on to decode how brat controls these genes. They find that Brat binds to specific sequences in the 3´UTR of both the zelda and dpn RNAs. Presumably due to ten Brat-target motifs in zelda and four in dpn, the negative regulation of zelda is more sensitive to Brat levels. This notion is partly verified by analysis of point-mutants of brat, and mis/overexpression of wt brat and mutant brat constructs. The negative regulation of translation by Brat onto the 3´UTR sequences can be re-capitulated also on a heterologous GFP construct in S2 cells.
I think this study provides an impressive push towards decoding the molecular underpinning of the brat brain overgrowth phenotype, and given the interest in TRIM proteins in many systems, including mammals, I think the story should be of broad interest. I have a few issues that may help strengthen the story:

Major issues:
1) Their transcriptome data, showing up-regulation of zelda and dpn mRNAs, point to a model where Brat binding results in degradation of zelda and dpn mRNAs. Is this the only way Brat acts? Or does Brat binding somehow inhibit mRNA nuclear export, ribosome docking and/or elongation?

> By using String proteomics database (https://string-db.org/), we analyzed all Brat's putative partners (with minimum confidence set to "medium": 0.400; 44 partners in total) described in the literature (Suppl. Table String Analysis For Reviewers, below in this word file). These included the other Neuroblast asymmetrically segregated factors Miranda, Pros, Numb and Pins, as well as Nanos and Pum, well described interactors of Brat. Importantly however, none of them had functions connected to mRNA export, ribosome docking or ribosome elongation. We could further

confirm that Brat mediated direct changes in its targets' mRNA level by qPCR in *brat*-deficient mINPs (new Fig6e). Whether Brat would act in the other post-transcriptional regulation mechanisms suggested by the reviewer is an intriguing possibility but would require a whole new set of experiments beyond our expertise.

2) What are the downregulated genes identified in the transcriptome analysis? They do not comment on this. They should show table of these genes.
> We've added the list of downregulated genes from our transcriptome analysis in new Supplemental Figure S1f.

3) In the same vein: What is the basis for increased proliferation in brat, Zelda and dpn RNAi or mutants? Increased CycE, Stg or E2f1, decreased Dap? Amongst the top upregulated genes, I could not recognize any clear cell cycle gene. Are they showing up in the downregulated genes?

> We followed reviewer's suggestion and monitored changes in CycE, Stg, E2f1 and Dap happening in INPs 24h following *brat* RNAi (FACS-sorted by GFP with similar settings than our RNA-Seq). Interestingly, CycE, Stg and E2F1 levels were unchanged while Dap levels were higher in brat-deficient cells (new Supplemental Fig. S1g). These results indicate that brat-deficient tumor initiating cells are rather blocked in their cell cycle at this stage compared to wild-type INPs. This is in line with previous observations that 24h-induced brat Type II NB clones showed less progeny (Bowman et al., 2008) and that their progeny is cell cycle delayed (Lee et al., 2006). By contrast, CycE, Stg and E2F1 were upregulated and dap unchanged in cells sorted from established *brat* tumors (7-days old larvae, without tubulin>Gal80^{TS}) (new Supplemental Figure S1g). Unlike *brat* RNAi (after 48h of RNAi), *zld* and *dpn* RNAi type II NB lineages are under-proliferative.

4) What is the phenotype of the brat[G774D] mutant? There is still repression of zld but dpn is ectopically expressed. But what is the consequence of this with regards to the Type II lineage development? PH3-labeling? Clone size?

> We further characterized *brat^{G774D}* mutant phenotype by following and quantifying advanced (72h) MARCM clones (new Supplemental Figure S8f-g). While, as expected, *brat^{G774D}* clones showed significantly more Dpn+ cells (interestingly, the average difference (3 cells) matches the number of Dpn- imINPs found in WT clones), these cells were not more PH3+ than WT mINPs and their overall cell numbers showed only a modest but non-significant increase (new Supplemental Fig. S8g). Similarly to WT, *brat^{G774D}* INPs gave rise to smaller Dpn-negative and Ase-positive cells (Supplemental Fig. S8e, new Supplemental Fig. S8f). Overall, despite their initial inability to repress dpn, *brat^{G774D}* INPs seem to behave similarly to WT with respect to their proliferative ability. We have added the following statement in the main text: "Importantly, these cells were not more proliferative than WT mINPs and the overall *brat^{G774D}* clone cell numbers were comparable to WT (suppl. Fig. S8f,g)."

Minor issues:
5) Abstract: Drosophile should be Drosophila.
> This has been corrected.

6) Introduction, 2nd paragraph: Drosophila NBs also come in the Type 0 flavor (PMIDs 15593370, 19945380, 25073156, 25171415).
> The reviewer is right; Type 0 NBs are now mentioned in the introduction.

7) I think the model in the supplement (S8) could be moved into the final main figures. I think their data presented here, combined with previous results, justify highlighting this model inside the main paper.
> We've followed reviewer's suggestion and transferred our model to Fig. 6 (now Fig. 6h).

8) In several figure legends (main text and suppl) Granyhead should be Grainy head or Grh. Also in the main text: Grainyhead should be Grainy head or Grh.
> This has been corrected.

9) Supplemental Figure S4e: Maybe help the reader along by outlining the green lineages in the panels to help show that V5 staining is lost in the zld-RNAi expressing lineages.

> We thank the reviewer for this suggestion. NB lineages have been outlined in all three panels of Supplemental Figure S7e (formerly S4e).

10) Pages 8-9: I do not agree with the notion of "active" and "inactive" zelda transcripts. What they have are two different splice variants. If they want to push the notion of active-inactive, they should use RNAi directed against each isoform separately, and/or use Crispr/Cas9 to mutate each isoform separately. I understand that previous studies have pointed to these roles of zelda isoforms, but this was in a different system.
> We agree with the reviewer and therefore performed isoform-specific RNAi against Zelda. We generated three UAS-shRNA transgenic lines directed against Zld-RD (presumably inactive) and one line against Zld-RB (presumably active). While *zld-RB* specific shmiR led to rescued *brat* tumor, none of the three *zld-RD* shmiRs did. Interestingly, *zld-RD* specific shmiRs instead showed a mild positive effect on tumor growth (new suppl. SFig 5a-c). Together with the rescue experiment performed with *zld-RB* (in new suppl. SFig 4a), these results indicate that the *zld-RB* isoform is key for proliferative ability of type II NB lineages, while *zld-RD* is dispensable.

11) Figure 4c-d: In the EMSA with Brat and Zelda RNA, why do the bound complexes migrate at different positions depending on the amount of Brat protein?
> We think that Brat can bind with variable affinities to the different binding sites on *zld* RNA, and higher concentrations of Brat could reach more than one site per RNA molecule, therefore, shifting its molecular weigh higher. Of note, this was observed previously with the same method in a previous paper investigating the interaction between Brat and *klu* or *kni* RNA (Loedige et al., 2015).

12) There are a bunch of typos throughout, and the manuscript would benefit from some closer scrutiny.
> We have carefully re-checked our manuscript and tried to remove as many typos as possible.

2nd Editorial Decision 25 September 2017

Thank you for the submission of your revised manuscript to our journal.
We have meanwhile received a complete set of reviews from all referees, which I include below for your information.

As you will see, the referees are very positive about the study and suggest overall only minor changes to clarify text and figures. However, referee 1 has some remaining concerns regarding the expression of Zelda in neuroblasts and mINPs that should be addressed and discussed in the most appropriate manner.

I look forward to seeing a final version of your manuscript as soon as possible.

****
REFEREE REPORTS

Referee #1:

The Reichardt et al. study is a revised version of a previously peer-reviewed manuscript. The authors made significant effort to address many of the concerns raised during the first round of review. This current version is much improved. The reviewer has a number of additional concerns that should be addressed by words if possible prior to accepting this study for publication in EMBO Rep.

1. The authors carried out additional experiments and provided additional data to strengthen their proposed model where distinct thresholds of Brat expression contribute to the expression patterns of Zld and Dpn in neuroblasts and in mINPs. However, their results do not rule out the possibility that zld is transcriptionally inactivated in mINPs. As a matter of fact, the FISH image in Supplemental Figure S7C shows little to no zld-RB transcripts in non-neuroblast cells both inside as well as outside of the dotted area. Thus, the authors must take their own data into account, and propose an alternative model where transcriptional silencing might contribute to lack of Zld-RB expression in

mINP.

2. In the same Supplemental Figure S7C, the signal for zld-RD inside the dotted area appears to be non-specific background staining. This is particularly evident when one examines the positive speckles of FISH signals located at the bottom left and the bottom right just outside of the dotted area. In addition, seeing positive zld-RD mRNAs does not automatically indicate that Zld-RD protein is indeed expressed in these iINPs. The authors should either confirm that Zld-RD protein is indeed detected in these cells, or they should remove this line of data from their text including the discussion section.

3. Along the same subject of Zld-RD, I looked up the paper that the authors cited, and found that the authors mis-interpret the published data as described on page 15. The published study actually showed that ZLD-PA and ZLD-PD like do not form a heterodimer, and that ZLD-PD likely interacts with a necessary co-factor for ZLD-PB keeping it from being recruited to DNA instead. The authors should revise their statement or come up with an alternative interpretation.

4. The manuscript needs a ton of copy editing.
a) Gene names need to be italicized.
b) When first introduced in the body of the manuscript Brat and Zld must be introduced as Brain tumor and Zelda.
c) The figure legends are clearly remnants of a previous version. Figure 3 g-h are no longer in the current figure. Figure 5 a- c is showing dpn but refers to zld. Figure 5 f states there is UAS-brat, but I don't see that in the figure as currently constructed. Fig 3b says it is in type II NB lineages, but the dpn shmir is in INPs. This certainly needs to be noted in the figure legend.

5. In Figure 4, mut 3-5 for Brat binding sites is misleading. It should be referred to as deletion 3-5.

6. In Figures 4 and 5, some of the panels are nearly invisible. zld9 mut 8-10, dpn 3, etc. Also, it appears that Brat still binds to the dpn 2 and 3 regions even when sites are mutated. This suggests they may be missing sites. Therefore, it is possible that the Brat mediated repression is actually more influential than suggested by the S2 assays shown in Figure 5d.

7. In the figure legend for Figure 2, they also state that this is "quantification" of brain sizes although the western shown is not quantitative. While convincing, it is certainly overexposed when it comes to the loading controls

Referee #2:

I have reviewed the revised version of the manuscript provided by the Knoblich lab. All my previous concerns were sufficiently addressed with the following minor exception:

I suggest to split the channels in Supplemental Figure S2; it is very difficult to discern the cyan from the green signal.

Other than that, I am enthusiastic about the revised manuscript.

Referee #3:

The authors have addressed all of my major concerns, and I think these changes have improved the manuscript.

2nd Revision - authors' response 31 October 2017

Referee #1:

The Reichardt et al. study is a revised version of a previously peer-reviewed manuscript. The authors made significant effort to address many of the concerns raised during the first round of

review. This current version is much improved. The reviewer has a number of additional concerns that should be addressed by words if possible prior to accepting this study for publication in EMBO Rep.

1. The authors carried out additional experiments and provided additional data to strengthen their proposed model where distinct thresholds of Brat expression contribute to the expression patterns of Zld and Dpn in neuroblasts and in mINPs. However, their results do not rule out the possibility that zld is transcriptionally inactivated in mINPs. As a matter of fact, the FISH image in Supplemental Figure S7C shows little to no zld-RB transcripts in non-neuroblast cells both inside as well as outside of the dotted area. Thus, the authors must take their own data into account, and propose an alternative model where transcriptional silencing might contribute to lack of Zld-RB expression in mINP.

The reviewer is right. We cannot exclude additional transcriptional regulation of Zld-RB in INPs. We addressed this in the Discussion section:
"*Importantly, our data do not exclude that pre- or co-transcriptional regulation of these factors would occur in parallel of Brat-mediated post-transcriptional control.*"

2. In the same Supplemental Figure S7C, the signal for zld-RD inside the dotted area appears to be non-specific background staining. This is particularly evident when one examines the positive speckles of FISH signals located at the bottom left and the bottom right just outside of the dotted area. In addition, seeing positive zld-RD mRNAs does not automatically indicate that Zld-RD protein is indeed expressed in these iINPs. The authors should either confirm that Zld-RD protein is indeed detected in these cells, or they should remove this line of data from their text including the discussion section.

We do believe that our *zld-RD* FISH signal is specific (and is further supported by our transcriptome data), but agree with the reviewer, we indeed have no proof that Zld-PD protein is expressed in NB progeny. We now therefore firmly state this fact in the Results section:
"*Importantly however, whether zld-RD is actually translated into functional proteins in these cells would remain to be explored*"

And in the Discussion section (changes underlined):
"*First, although Zld-PD cannot activate transcription and lacks the relevant domains [39], <u>its RNA</u> is expressed in the NB progeny and could <u>potentially</u> antagonize Zld-PB function. <u>Whether Zld-PD is actually translated into functional proteins in these cells would remain to be explored.</u> However, it has been shown that co-expression of Zld-PA (identical to PB) and Zld-PD significantly reduced gene expression, demonstrating that Zld-PD acts dominantly to suppress Zld-mediated transcriptional activation.*"

3. Along the same subject of Zld-RD, I looked up the paper that the authors cited, and found that the authors mis-interpret the published data as described on page 15. The published study actually showed that ZLD-PA and ZLD-PD like do not form a heterodimer, and that ZLD-PD likely interacts with a necessary co-factor for ZLD-PB keeping it from being recruited to DNA instead. The authors should revise their statement or come up with an alternative interpretation.

We have revised our statement accordingly.
(The formation of non-functional multimer was one possible explanation, but there was no evidence for this. Thus the authors rather explain that by competition of the two isoforms for interaction with cofactors required to activate transcription.)

4. The manuscript needs a ton of copy editing.
a) Gene names need to be italicized.
b) When first introduced in the body of the manuscript Brat and Zld must be introduced as Brain tumor and Zelda.
c) The figure legends are clearly remnants of a previous version. Figure 3 g-h are no longer in the current figure. Figure 5 a- c is showing dpn but refers to zld. Figure 5 f states there is UAS-brat, but I don't see that in the figure as currently constructed. Fig 3b says it is in type II NB lineages, but the dpn shmir is in INPs. This certainly needs to be noted in the figure legend.
This was changed accordingly.

5. In Figure 4, mut 3-5 for Brat binding sites is misleading. It should be referred to as deletion 3-5.
We agree and corrected this accordingly (applied to zelda3 and zelda4 fragments).

6. In Figures 4 and 5, some of the panels are nearly invisible. zld9 mut 8-10, dpn 3, etc. Also, it appears that Brat still binds to the dpn 2 and 3 regions even when sites are mutated. This suggests they may be missing sites. Therefore, it is possible that the Brat mediated repression is actually more influential than suggested by the S2 assays shown in Figure 5d.
We agree with the reviewer and added the following statement in the Results part:
"Consistently, mutations of the motif sites in fragment 1 abolished Brat-NHL binding (Fig 5c). Interestingly however, mutations of these motifs in fragment 2 and 3 did not prevent Brat-NHL binding, suggesting the existence of alternative binding specificities."

7. In the figure legend for Figure 2, they also state that this is "quantification" of brain sizes although the western shown is not quantitative. While convincing, it is certainly overexposed when it comes to the loading controls
We agree with the reviewer and now provide a less exposed picture of the loading controls of Figure 2 instead of the previously overexposed ones.

Referee #2:

I have reviewed the revised version of the manuscript provided by the Knoblich lab. All my previous concerns were sufficiently addressed with the following minor exception:

I suggest to split the channels in Supplemental Figure S2; it is very difficult to discern the cyan from the green signal.

We followed reviewer's suggestion: First, an individual Deadpan channel is provided for each panel. Seconds, we changed the colors to display a more comprehensive merged picture (GFP is now in white, Deadpan in red, Asense in green).

Other than that, I am enthusiastic about the revised manuscript.

Referee #3:

The authors have addressed all of my major concerns, and I think these changes have improved the manuscript.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jürgen Knoblich
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44188V2

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Supplementary Experimental Procedures / Page 1 / Statistics
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Supplementary Experimental Procedures / Page 1 / Statistics
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Supplementary Experimental Procedures / Page 1 / Statistics
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Supplementary Experimental Procedures / Page 1 / Statistics
For animal studies, include a statement about randomization even if no randomization was used.	Supplementary Experimental Procedures / Page 1 / Statistics
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Supplementary Experimental Procedures / Page 1 / Statistics
4.b. For animal studies, include a statement about blinding even if no blinding was done	Supplementary Experimental Procedures / Page 1 / Statistics
5. For every figure, are statistical tests justified as appropriate?	Supplementary Experimental Procedures / Page 1 / Statistics
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Supplementary Experimental Procedures / Page 1 / Statistics
Is there an estimate of variation within each group of data?	Supplementary Experimental Procedures / Page 1 / Statistics
Is the variance similar between the groups that are being statistically compared?	Supplementary Experimental Procedures / Page 1 / Statistics

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Manuscript / Page 19-20 / Experimental Procedures / Antibodies

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* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Manuscript / Page 19 / Experimental Procedures / Fly strains, RNAi and Clonal analysis
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance to the ARRIVE guidelines and NIH and MRC recommendations.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Suppl.Table S1, Suppl.Fig.S1f
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20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA